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TITLE: Novel Role of Merlin Tumor Suppressor in Autophagy and its Implication in  
Treating NF2-Associated Tumors

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14. ABSTRACT The overall goal of the proposed research is to elucidate the novel function of Merlin in autophagy, a cellular catabolic pathway implicated in tumorigenesis, and to control the Merlin-mediated tumorigenesis by modulating cellular autophagy. We have recently demonstrated that Merlin binds an autophagy regulator protein, and that loss of Merlin leads to attenuated autophagy as well as enhanced hypoxia or metabolic stress in the three-dimensional (3D) microenvironment in culture, a condition known to accelerate tumor formation. Moreover, this elevated level of metabolic stress is suppressed by rapamycin, an autophagy inducer. Therefore, we hypothesize that Merlin normally suppresses tumorigenesis in part by activating autophagy, and that this new role of Merlin in autophagy could be a target for therapeutic intervention for NF2-associated tumors. To test this hypothesis, we precisely evaluated the interaction of Merlin with autophagy-related proteins, including LC3, and Unc51.1/Atg1. This analysis revealed that the interaction of Merlin with LC3 and Unc51/Atg1 is significantly upregulated upon autophagy induction. In addition, we have successfully developed the cell culture system to evaluate autophagy-inducing ability of Merlin mutants. This analysis revealed that at least one point mutant Merlin/K79E specifically abolished the autophagic activity. We will continue to advance our study to delineate the role of Merlin-mediated autophagy in the regulation of tumorigenesis.					
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## Introduction:

We have recently found that the tumor suppressor Merlin promotes autophagy, a cellular clearance system that is responsible for removing old proteins or damaged organelles within cells, and thus help mitigate the risk of tumor formation. Moreover, we found that the cellular stress caused by loss of Merlin function was effectively suppressed by rapamycin, an autophagy-inducing compound. Therefore, we hypothesize that Merlin normally suppresses tumorigenesis in part by activating autophagy, and that this new role of Merlin in autophagy could be a target for therapeutic intervention for NF2-associated tumors. To test this hypothesis, we will evaluate the interaction of Merlin with autophagy-related proteins (i.e., LC3, Unc51.1/Atg1). We will also analyze how Merlin mutations found in NF2 patients may affect the autophagy-inducing activity of Merlin protein. These analyses are expected to provide new insight into the role of Merlin in autophagy and to generate the therapeutic strategy for NF2.

## Body:

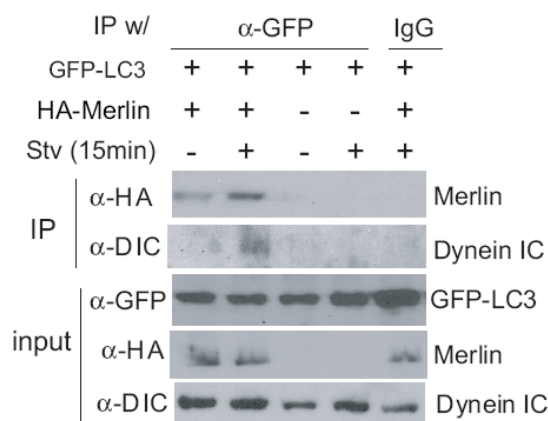
### Interaction studies of Merlin and autophagy-related protein (Aim 1)

#### 1a. Preparation of expression constructs

We have finished constructing all the mammalian expression constructs necessary to carry out this interaction study, which include GFP-tagged LC3, myc-tagged Merlin, HA-tagged Merlin, and myc-tagged Unc51.1/Atg1.

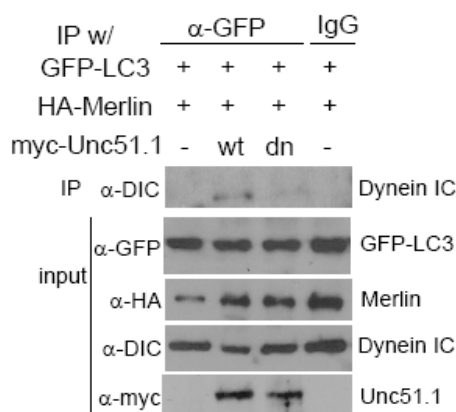
#### 1b. Transfection and immunoprecipitation experiments

Using these reagents, we transfected mammalian cell line (HEK293T) to carry out immunoprecipitation (Figs.1, 2, 3).



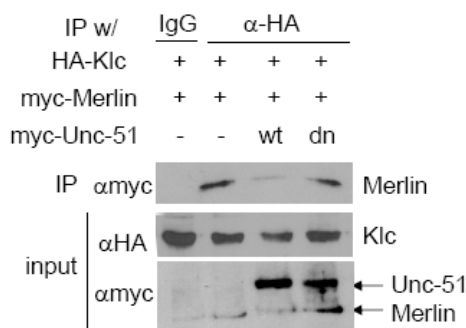
**Fig.1** Merlin assembles with LC3 and Dynein upon autophagy induction.

HEK293T cells were transfected with the indicated vectors that express Merlin, LC3, and Dynein. Cell lysates were immunoprecipitated (IP) and analyzed by Western blots.



**Fig.2** Merlin-Dynein interaction is positively regulated by Unc51.1/Atg1 kinase activity.

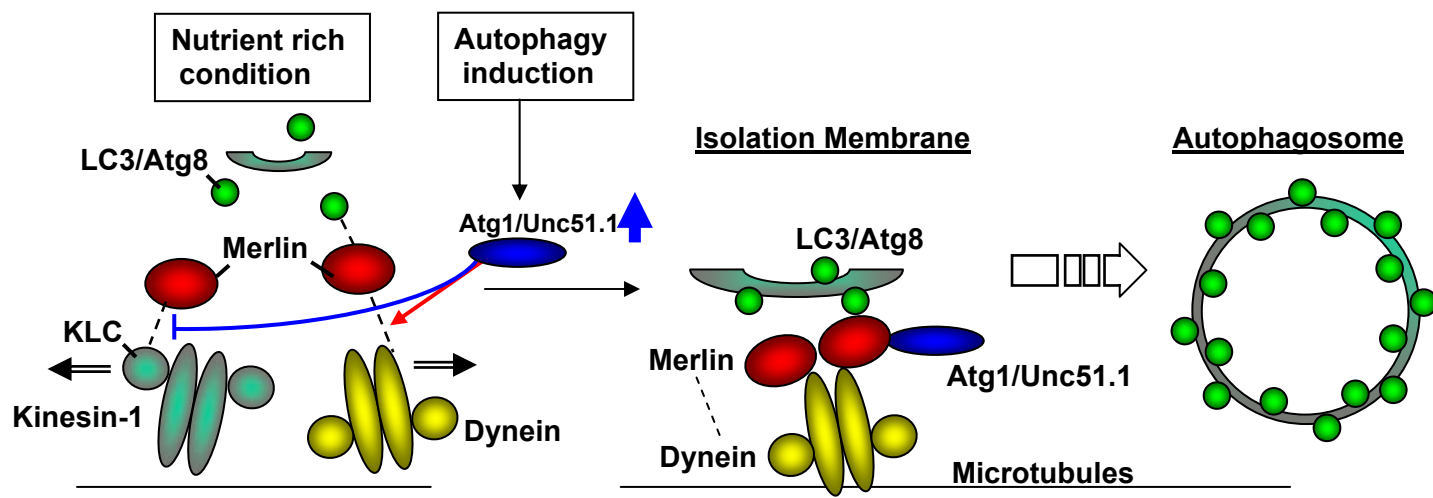
HEK293T cells were transfected with the indicated vectors that express Merlin, LC3, and Dynein. Cell lysates were immunoprecipitated (IP) and analyzed by Western blots.



**Fig.3** Merlin-kinesin interaction is negatively regulated by Unc51/Atg1 kinase activity.

HEK293T cells were co-transfected with the indicated plasmids and the resulting cell lysates were analyzed by immunoprecipitation with anti-HA or the control IgG, followed by Western blots using anti-myc and anti-HA.

#### 1c. Data analysis for 1a and 1b



#### Hypothetical scheme

Based on the data in Figs. 1-3, we hypothesized that Merlin serves as an adaptor protein that links LC3 autophagy protein and Dynein motor, which helps deliver LC3 towards future autophagic membranes. Upon autophagy induction by nutrient starvation or rapamycin, Unc51.1/Atg1 kinase activity is upregulated and induces the association of Merlin and DIC, but inhibits the association of Merlin and kinesin light chain (KLC), which helps autophagic membrane to mature into double membrane structures.

#### 1d. Setting up mouse crosses to prepare MEFs

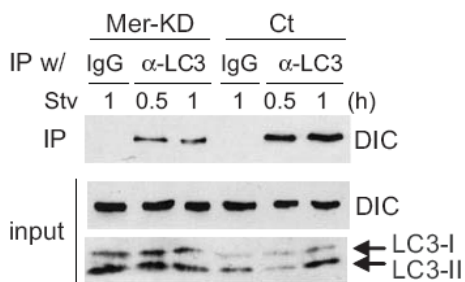
Unc51.1/Atg1 heterozygous mice were intercrossed and both wild-type and homozygous mutant MEFs were successfully prepared.

#### 1e. Preparation of MEFs

Unc51.1(-/+);GFP-LC3/+ mice crossed with Unc51.1(-/+) mice had provided GFP-positive MEFs and GFP(-) MEFs with an expected ratio (approx. 1:1). MEFs were successfully prepared from both populations.

#### 1f. Transfection of MEFs and interaction assays

MEFs were transfected with Merlin-knockdown construct and LC3-DIC interaction assay was done by immunoprecipitation (Fig. 4).



**Fig.4** Merlin knockdown attenuates the LC3–dynein complex formation in MEFs upon autophagy induction.

#### 1g. Data analysis for 1f

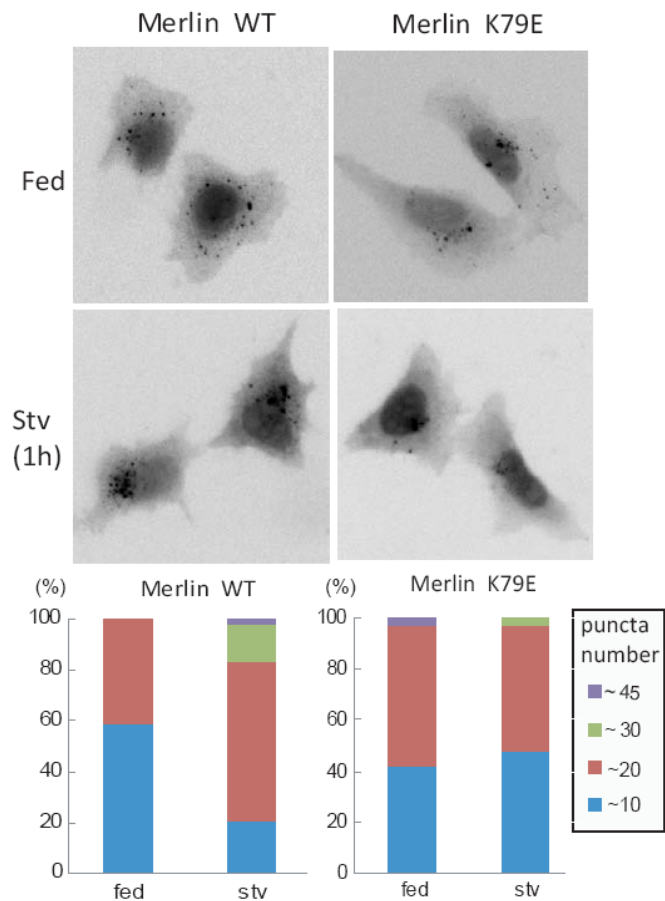
As expected from the IP experiments using heterologous expression system (Figs. 1-3), interaction of endogenous LC3 with DIC was significantly attenuated by loss of Merlin expression, indicating that Merlin serves as an adaptor protein that links LC3 and DIC.

#### Evaluation of autophagy activity of mutant Merlin (Aim 2)

##### 2a. Preparation of expression constructs

We have finished constructing all the mammalian expression constructs necessary to carry out this study, which include myc-tagged Merlin/WT, K79E, E270G mutants.

2b. Transfection and immunocytochemistry experiments



**Fig.5** HeLa cells were co-transfected with GFP-LC3 and mouse Merlin (either wild-type [WT] or K79E) and the autophagosomes were observed by fluorescence microscopy before (Fed) and after nutrient starvation (Stv) in Earle's balanced salt solution (EBSS) for 1 h. Scale bar: 10  $\mu$ m. The numbers of GFP-LC3-positive puncta per cell were scored and the percentages of cells that contained the indicated ranges of the puncta numbers (~10, up to 10 puncta; ~20, from 11 up to 20 puncta; ~30, from 21 up to 30 puncta; ~45, from 31 up to 45 puncta) are plotted in the graphs.

2c. Data analysis for 2a and 2b  
We found that Merlin/K79E expression dominant-negatively suppresses autophagy induction upon starvation.

**Key Research Accomplishments:**

- Merlin promotes autophagic membrane formation by linking autophagy-related protein LC3 and dynein motor.
- NF2-associated mutation Merlin/K79E inhibits autophagy induction.

**Reportable Outcomes:**

- The above data was presented at the poster session at Childrens' Tumor Foundation (CTF) annual Meeting held at Jackson Hole WY (June 2011).
- Dr. Donald Jhung Jr. obtained Ph.D. as a result of his studies supported by this award (June 2011).
- CTF-drug discovery initiative research award was applied and the PI obtained this award (Oct. 2011).
- A new research scholar, Ms. Yuki Hirota, has been employed (June 2011~).
- Poster session describing this work presented by Ms. Yuki Hirota was selected No. 1 in the annual poster session presentation competition held at City of Hope (Jan. 2012).
- PI presented this work at the Special symposium on Autophagy; Health and Disease, held at City of Hope (Mar. 2012).

**Conclusion:**

By extending the analyses presented in the initial grant proposal, we have successfully confirmed the novel role of Merlin in promoting autophagy. We showed that Merlin is a part of multi-protein complex that serves as a scaffolding machinery to promote autophagic membrane assembly. At least one of the NF2-associated mutation was found inhibitory to autophagy induction. Further analyses need to be done to delineate the role of Merlin in autophagy. In particular, we will need to evaluate the relevance of autophagy in the NF2-relevant cell line, such as schwannoma cell lines from NF2 patients. Dr. Marco Giovannini (at University of Southern California / House Ear Institute) has a collection of such cell lines, and we have initiated the collaborative work to address this issue.

**References:**

None

**Appendices:**

None

**Supporting Data:**

Five figures and one schematic diagram included in the body of this report.